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Microbial community analysis of Lake Chillisquaque, a small water system in Central Pennsylvania

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by

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ABSTRACT

Cyanobacteria are photosynthetic organisms that require the absorption of light for the completion of photosynthesis. Cyanobacteria can use a variety of wavelengths of light within the visible light spectrum in order to harvest energy for this process. Many species of cyanobacteria have light-harvesting proteins that specialize in the absorption of a small range of wavelengths of light along the visual light spectrum; others can undergo complementary chromatic adaptation and alter these light-harvesting proteins in order to absorb the wavelengths of light that are most available in a given environment. This variation in light-harvesting phenotype across cyanobacteria leads to the utilization of environmental niches based on light wavelength availability. Furthermore, light attenuation along the water column in an aquatic system also leads to the formation of environmental niches throughout the vertical water column. In order to better understand these niches based on light wavelength availability, we studied the composition of cyanobacterial genera at the surface and depth of Lake Chillisquaque at three time points throughout the year: September 2009, May 2010, and July 2010. We found that cyanobacterial genera composition changes throughout the year as well as with physical location in the water column. Additionally, given the light attenuation noted throughout the Lake Chillisquaque, we are able to conclude that light is a major selective factor in the community composition of Lake Chillisquaque.

INTRODUCTION

Cyanobacteria

Cyanobacteria are a diverse group of photosynthetic prokaryotes distributed throughout the world in both aquatic and terrestrial ecosystems. They have adapted to a range of both temperate and extreme environments and are often characterized by their ability to undergo oxygenic photosynthesis; in fact, it is thought that 20-30% of primary production on Earth is a result of the conversion of solar energy into chemical energy by cyanobacterial species (Pisciotta et al. 2010). Because of this ability, cyanobacteria are hypothesized to be largely responsible for the initial oxygenation of our atmosphere (Stainer and Cohen-Bazire 1977). Furthermore, the endosymbiotic theory suggests that endocytosis of cyanobacteria by an early eukaryotic cell provided a symbiotic relationship between early eukaryotes and cyanobacteria, resulting in the eventual evolution of chloroplasts (McFadden 2001). Cyanobacteria, therefore, are important in the biogeochemistry of the planet, affecting both the evolution and the maintenance of species.

Cyanobacterial Cycling

Within a lake ecosystem, changes in community structure are expected throughout the year. In dimictic lakes, temporal variations in community structure are often due to the alteration between stratification and mixing in the water temperature throughout the year. For example, for most dimictic lakes, cooler winter months (November to late March) show patterns of consistent temperature throughout the water column, allowing for mixing of bacteria, nutrients, and dissolved particles throughout the water column. Warmer summer months (April to October) lead to stratifications in the water column based on temperature creating the epilimnion,

metalimnion, and hypolimnion; stratification of these water layers prevents bacteria, nutrients and dissolved particles from mixing into different regions within the water column (Wetzel 2001). Dissolved nutrients such as nitrogen and phosphorous – useful to many species of cyanobacteria – also follow these patterns of stratification and mixing, and therefore play important roles in the changes in community structure observed in lake ecosystems throughout the year (Wetzel 2001). It is these patterns of nutrient availability as well as lake mixing and stratification that lead to cycling patterns of the cyanobacterial community throughout the year – cyanobacteria play a very small role in ecosystem biomass during winter months, and undergo high rates production in the late summer months, between July and September (Wetzel 2001).

Genera of Cyanobacteria

Among the cyanobacteria, there is great diversity in species morphology. Genera are often distinguished based on cell type – unicellular or colonial – but motility, method of reproduction, and cell size can also be analyzed in order to classify genera (Stainer and Cohen-Bazire 1977). Additionally, some genera of cyanobacteria form heterocysts, specialized cells that fix atmospheric nitrogen (N_2), making ammonia (NH_3) more available for biosynthetic activities (Litchman and Klausmeier 2008). Importantly, pigmentation and the composition of light-harvesting structures, known as phycobilisomes, also differ among genera of cyanobacteria (Haverkamp et al. 2009). Given the diversity of environments inhabited by cyanobacteria, these phenotypic variations allow for optimal survival of these species across a range of habitats.

Molecularly, the use of the 16S rRNA gene region has also allowed researchers to distinguish among genera of cyanobacteria. The 16S rRNA gene is a non-protein coding region of the bacterial chromosome that is highly conserved among bacteria; however, it contains

several highly variable regions that allow researchers to distinguish among genera (Haverkamp et al. 2009; Nübel et al. 1997). Other regions of the genome, described as genomic islands, are much less conserved and often include regions that encode for structural proteins; as a result, they are subjected to more rapid rates of evolution through deletion and addition mutational events (Scalan et al. 2009). Thus, compared to the 16S rRNA region, genomic islands are hypothesized to be necessary for adaptation of cyanobacteria to specific environments. For example, in *Synechococcus*, the region of DNA encoding the rod of the phycobilisome, a structure used to absorb light from the environment, is homologous among many species of *Synechococcus* (Scalan et al. 2009). Yet within this sequence various mutational events have occurred over time yielding a variety of phycobilisome phenotypes. Certain phenotypes give these species of *Synechococcus* adaptive advantages in light-harvesting ability making these species of *Synechococcus* better fit for survival in specific environmental niches (Scalan et al. 2009). However, in studies exploring the diversity of species, it is favorable to use the 16S rRNA region – or other highly conserved regions of the genome – to distinguish among species, because these regions are most consistent within the phylum cyanobacteria, yet they are sensitive to small nucleotide difference that indicate variation among species (Ernst et al. 2003; Woese 1987).

Phycobilisome Structure and Regulation

Synechococcus is not unique in its use of phycobilisomes. In fact, phycobilisomes are the functional units consisting of rod and core regions that all cyanobacteria use to absorb light from the environment and funnel it to photosystem II for the completion of photosynthesis (Montgomery 2007; Grossman et al. 1993). In order to absorb environmental light, each phycobilisome is comprised of phycobiliproteins – allophycocyanin, phycocyanin,

phycoerythrin, and phycoerythrocyanin – which absorb specific wavelengths of light from the environment (Figure 1). Each of these phycobiliproteins is encoded by separate genes that are differentially expressed depending upon nutrient availability and light quality, specifically the wavelength of available photons (Grossman et al. 2001). Most important to our analysis are phycocyanin, responsible for the absorption of red light, and phycoerythrin, responsible for the absorption of green light. All species of cyanobacteria contain functional genes for phycocyanin; those cyanobacteria able to harvest green light also include functional genes for phycoerythrin (Figure 1 and 2) (Tandeau de Marsac 1977).

For some cyanobacteria, light triggers several structural changes within the organism. Depending on the wavelength of light – ranging from red light to green light – certain species of cyanobacteria are able to alter the composition of phycobilisome rod structures in order to optimally absorb adequate levels of light; this process is known as complementary chromatic adaptation (CCA) (Figure 1) (Grossman et al. 1993). Species of cyanobacteria that are able to undergo CCA also contain functional genes for both phycocyanin and phycoerythrin within their genome; however, in contrast to species that contain phycoerythrin but are unable to undergo CCA, CCA-capable species are able to alter phycocyanin and phycoerythrin composition within the phycobilisome rod in response to changing light quality by altering the transcriptional rate of the phycobiliprotein genes (Figure 2). Species unable to undergo CCA cannot alter phycobiliprotein gene expression in this way (Tandeau de Marsac 1977). For CCA-capable species, under red light conditions, the gene *cpcB2A2* is expressed resulting in the production of phycocyanin containing phycobilisomes, which gives a green appearance to the cyanobacteria. Under green light conditions, the CCA-capable cyanobacteria instead appear red following the expression of the *cpeBA* gene, which alters outer rod composition to include the phycoerythrin

phycobiliprotein (Grossman et al. 2001). This variation in gene expression in the phycobilisomes is regulated by a phosphorelay system (Stowe-Evans et al. 2004).

According to the current signal transduction model, in the presence of red light the photoreceptor, RcaE, becomes phosphorylated, and starts a phosphorylation cascade by phosphorylating the response regulator RcaF, which then phosphorylates RcaC. The phosphorylation of RcaC inhibits the transcription of *cpeCDESTR*, which in turn prohibits transcription of *cpeBA*, and, therefore, halts the production of phycoerythrin (Figure 2). Conversely, phosphorylation of RcaC promotes transcription of *cpcB2A2*, and thus the production of phycocyanin phycobiliproteins. In green light, the phosphorylation of RcaE, RcaF, and RcaC does not occur; as a result, *cpeCDESTR* transcription is no longer inhibited, promoting the transcription of *cpeBA* and the production of phycoerythrin phycobiliproteins (Figure 2) (Stowe-Evans et al. 2004; Kehoe and Gutu 2006). Altering wavelengths of light does not cause the breakdown of phycoerythrin or phycocyanin, but rather, promotes the transcription of one type of phycobiliprotein operon over the other. Over time, this results in a higher concentration of one type of phycobiliprotein relative to another as older phycobiliproteins are replaced by newly synthesized phycobiliproteins (Tandeau De Marsac 1977).

Not all CCA-capable cyanobacteria respond to differing light quality in the same manner. Instead, chromatically adapting cyanobacteria can be clustered into three categories – group 1, group 2, and group 3 chromatic adaptors – each resulting in characteristic ratios of phycobiliprotein production relative to light quality. Group 1 species show little variation in phycobiliprotein composition given changing light quality; the ratio of phycoerythrin and phycocyanin produced remains close to 1 regardless of variation in red and green light availability. Group 2 species retain consistent phycocyanin production, while modifying the level

of phycoerythrin produced. Thus, despite changes in light wavelength, in group 2 species, the phycocyanin produced in red light versus green light remains close to 1 while the ratio of phycoerythrin produced in red light versus green light becomes less than 1. Finally, group 3 chromatic adaptors demonstrate altered concentrations of both phycocyanin and phycoerythrin upon variation in light conditions. The amount of phycocyanin produced in red light is almost two times that of the amount produced in green light; the ratio of phycoerythrin produced in red light compared green light, however, is less than one. Thus, phycoerythrin production decreases in red light and increases in green light, whereas phycocyanin production increases in red light and decreases in green light (Tandeau De Marsac 1977).

While the phenotypic plasticity of CCA is evolutionarily ideal for species – especially in fluctuating light environments – as previously noted, not all species of cyanobacteria exhibit this flexibility in light-harvesting structures. The majority of cyanobacterial species can be characterized as either phycoerythrin-rich or phycocyanin-rich and, thus, thrive in only environments in which green light or red light are available, respectively (Grossman et al. 2001). It is therefore possible that light environment could dramatically alter the community structure of cyanobacterial species and lead to the formation of well-defined niches structured around differing wavelengths of available light.

Niche Selection

A niche is a set of conditions and resources that an organism uses in a specific environment. Species in competition for valuable resources often exhibit an adaptive pattern of niche selection; commonly, this yields an ecosystem of highly defined niches in which organisms are well adapted to optimize the resources and conditions within their own specialized niche, though niche overlap between organisms does occur (Smith and Smith 2009). However, in

environments in which little competition exists among species, niche differentiation is less precise and coexistence of species requiring similar resources is more likely (Stomp et al. 2004).

Cyanobacteria provide a fascinating example of niche selection. Due to the variation in light wavelength required by different species, species with different phycobiliprotein composition occupy different environmental niches. Moreover, chromatically adapting species can alter their occupied niche for light wavelength based on the light available to them in the environment by altering their phycobiliprotein composition (Stomp et al. 2004).

Light Attenuation and Community Structure in Aquatic Environments

Given the likelihood of light-characterized niches for cyanobacterial species, in an aquatic ecosystem in which light attenuation changes with depth, it is reasonable to assume that cyanobacterial community composition will change as the wavelength of available light changes within the water column. Most light that hits the water's surface is reflected back into the atmosphere; of the light that does penetrate the water column, water effectively absorbs the wavelengths of light in the red spectrum (McArthur 2006). Thus, red light, with a relatively long wavelength of about 650 nm, is most readily available near the surface of the water column, but diminishes with increasing depth in the water column. Green light, with a shorter wavelength of approximately 510 nm, is also available near the surface of the water column, but given the increased light absorbance of long wavelengths and, thus, narrowing of the spectrum of light, with increased depth in the water column, green light becomes the dominant available wavelength of light at depth (Figure 3) (Stomp et al. 2007).

Other factors that alter the light absorption spectrum within the water column include components of the water column that Stomp and colleagues refer to as "background turbidity" –

dissolved organic matter and suspended particles, including other species of phytoplankton as well as sediment and detritus. An increase in overall background turbidity of an aquatic system increases the absorption of blue wavelengths in comparison to red wavelengths of light and therefore pushes the underwater available light spectrum closer to red; the absorption spectrum of water has the opposite effect in that with increased absorption, the light spectrum is shifted to the shorter, blue wavelengths. In this way, light absorption and background turbidity within the water column play dynamic roles in shifting the available light spectrum at depth to the intermediate, green wavelength (Figure 3) (Stomp et al. 2007).

Previous Research on Aquatic Community Structure

Niche selection has been described in large saline aquatic systems – for example, in the Baltic Sea. Studies of species of *Synechococcus* isolated from various locations and depths throughout the Baltic Sea have demonstrated the coexistence of phycocyanin-rich and phycoerythrin-rich *Synechococcus* species within the Batic Sea ecosystem (Haverkamp et al. 2008). This coexistence is possible because, though species of *Synechococcus* are all in competition for the available light resources, they vary in their light-harvesting phycobilisomes allowing them to utilize different regions of the light spectrum. Studies of Baltic Sea *Synechococcus* diversity have recorded a larger abundance of phycocyanin-rich *Synechococcus* within the upper 5 meters of the water column where red light is most available, as well as a greater abundance of phycoerythrin-rich *Synechococcus* species between the 5-15 meter region in the water column where green light predominates over red light (Haverkamp et al. 2008).

These environmental studies of cyanobacterial diversity based on light attenuation within aquatic ecosystems support studies on lab-completed competition experiments which model species competition based on light availability (Stomp et al. 2004). These studies predict that

species of phycocyanin-rich and phycoerythrin-rich cyanobacteria will be able to coexist in aquatic environments by stratifying themselves in different locations of the vertical water column based on their composition of phycobilisomes. Furthermore, these models predict that in the presence of cyanobacterial species able to undergo CCA, coexistence of CCA-capable and CCA-incapable species will occur. In this situation, species with the flexible CCA phenotype will alter phycobilisome composition in order to optimally absorb the wavelength of light not utilized by the cyanobacterial species with the fixed phenotype. For example, in an environment in which CCA-capable species coexist with phycoerythrin-rich cyanobacterial species, the CCA species will alter its phycobilisome composition in order to optimally absorb red light, the wavelength of light less utilized by phycoerythrin-rich species (Stomp et al. 2004).

Light Attenuation and Community Structure in Lake Chillisquaque

Our studies focus on smaller, freshwater aquatic systems, in which we have noted similar light attenuation patterns occurring at more shallow depths as compared to the Baltic Sea. Our studies focus on Lake Chillisquaque, which is a 165-acre reservoir on the middle branch of Chillisquaque Creek located 11 miles north of Danville, PA. The lake is currently used recreationally for fishing and boating. Since its creation in 1972 by Pennsylvania Power and Light as a source of backup cooling water for their Montour County power plant, it has also been impacted by agricultural runoff from the prevalent farming industry of the surrounding area (Figure 4) (PPL Corporation 2010).

Within Lake Chillisquaque, a dimictic lake, we expect to see variations in cyanobacterial community structure over time, due to known patterns of temporal cyanobacterial cycling. We expect to see a larger relative abundance of cyanobacterial species within the bacterial

community in warm, late summer months compared to cooler months of the year (Wetzel 2001). This will likely be due to variations in water temperature and nutrient availability over time.

In comparison to previous studies, we also expect our studies of this aquatic environments to reflect community structure and species stratification throughout the water column similar to those noted in competition modeling experiments and in the studies of the much larger Baltic Sea. In the small, central Pennsylvania, freshwater system of Lake Chillisquaque, we expect to see several variations in the diversity of cyanobacterial species based on phycobilisome composition. At the surface, we expect to see a greater abundance of species containing only phycocyanin compared to at depth. In comparison, at depth we expect species composition to be dominated by species that are known to be phycoerythrin-containing. It is also expected, however, that some phycoerythrin-containing species will still be found at the surface, in order to occupy the available niche for green light at the surface. CCA-capable species diversity will also change depending on the depth of sampling from the water sources sampled. Because the penetration of light wavelengths decreases as depth in the water column increases, it can be hypothesized that samples from depths will contain higher levels of chromatically adapting species than non-chromatically adapting species due to their ability to harvest a larger variety of available light; surface samples are less likely to have a high abundance of chromatically adapting species due to the consistent availability of all visible wavelengths of light.

Available light within the water column is, therefore, hypothesized to be a major selective factor for species composition at altered depths within the water column in small, freshwater ecosystems. Using microbial community analysis at various depths, we will test the hypothesis that light quality affects cyanobacterial community structure.

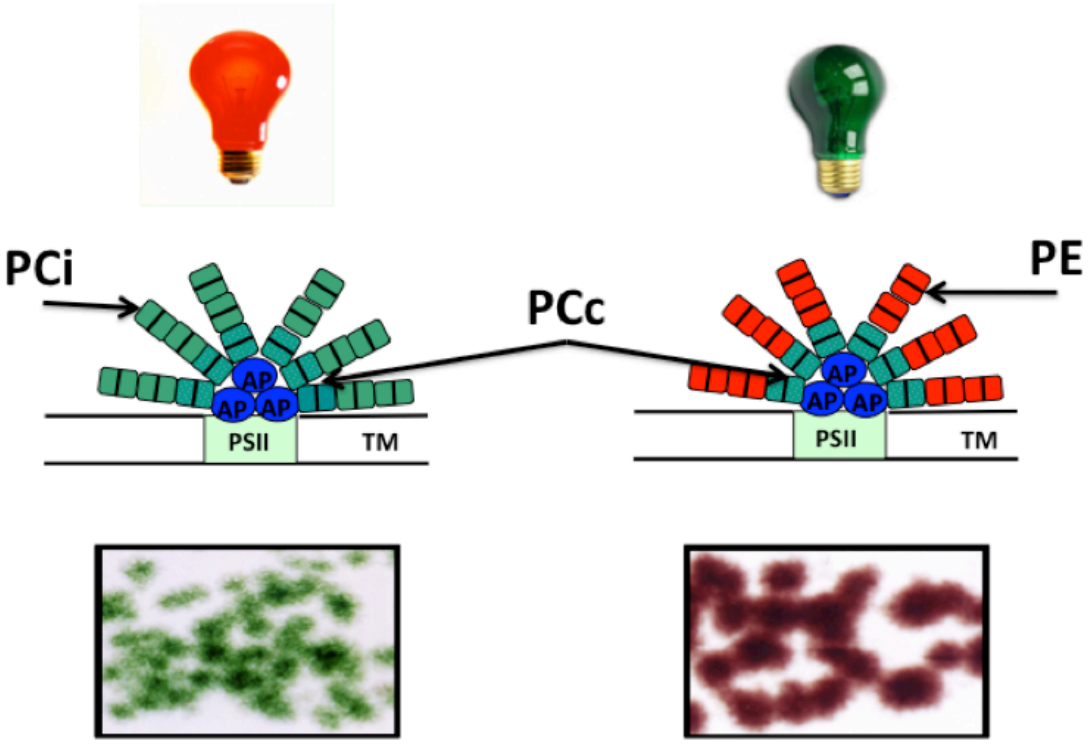

Figure 1. Complementary chromatic adaptation. Core structures of phycobilisomes consist of allophycocyanin (AP) and constitutive phycocyanin (PCc) phycobiliproteins. Rod structures contain either inducible phycocyanin (PCi) or phycoerythrin (PE) phycobiliproteins. In red light, the phycobiliproteins in the rod structure of the phycobilisome are comprised of PCi; cyanobacteria appear green. In green light, the phycobiliproteins in the rod structure of the phycobilisome are comprised of PE; cyanobacteria appear red. (Grossman et al. 2001).

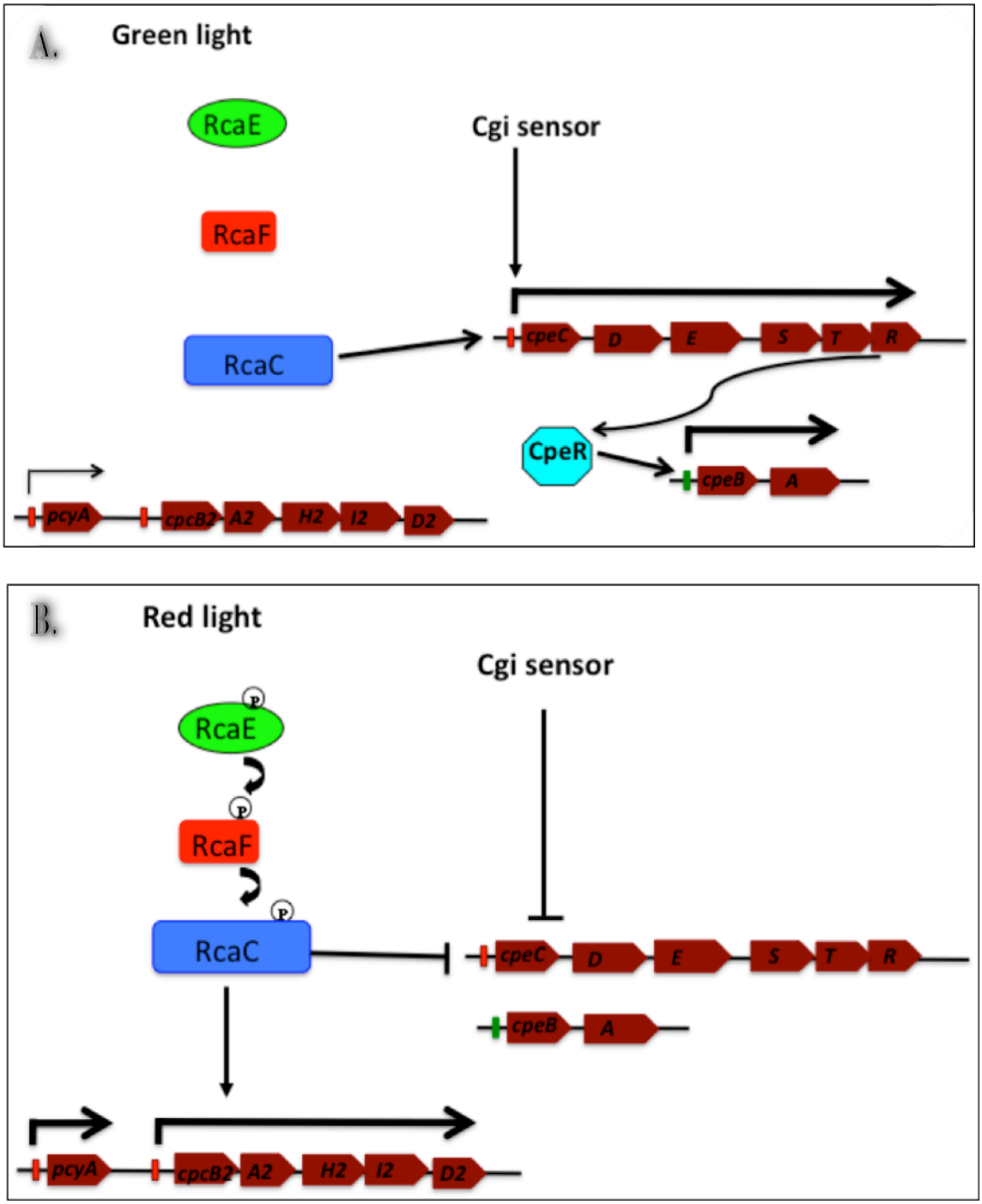

Figure 2. Phosphorelay signaling cascade controlling phycobilisome rod composition. A. In green light, unphosphorylated RcaC cannot inhibit the transcription of *cpeCDESTR*, promoting the transcription of *cpeBA*, the gene for phycoerythrin phycobiliproteins. B. In red light, a phosphorylation cascade of RcaE, RcaF, and RcaC inhibits the transcription of *cpeCDESTR*, and, therefore, the expression of phycoerythrin phycobiliproteins (Kehoe and Gutu 2006).

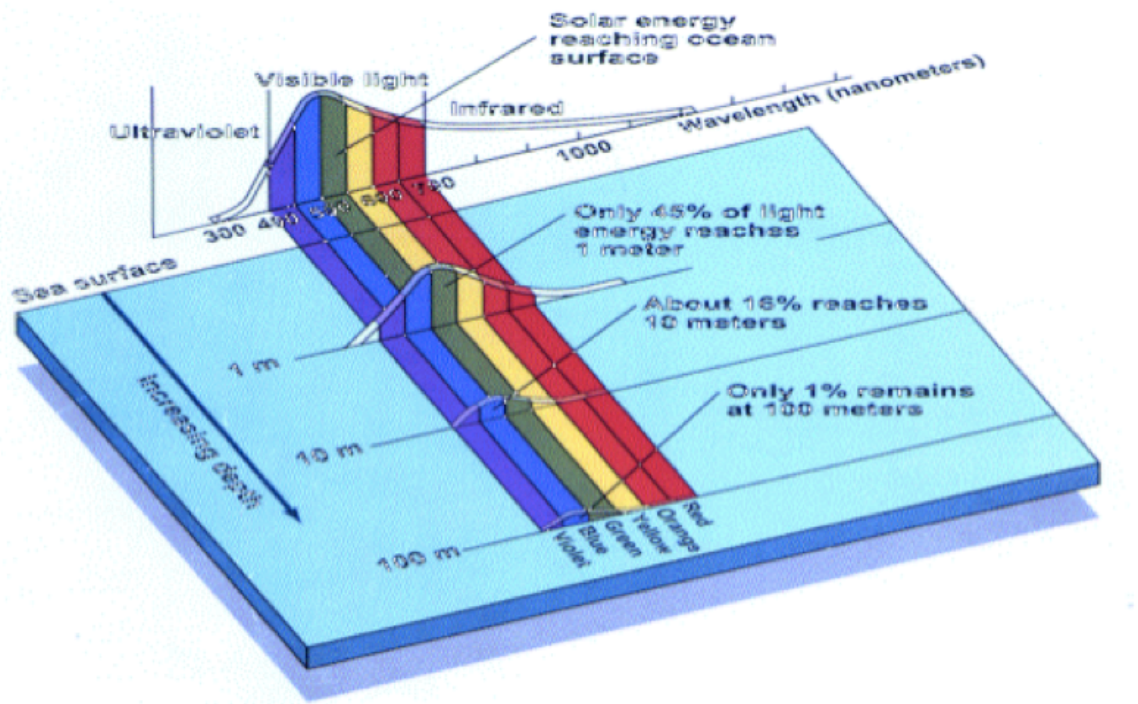

Figure 3. Light attenuation throughout the water column. The light spectrum is predicted to narrow to shorter wavelengths with increasing depth in the water column.

Figure 4. Lake Chillisquaque. A. View from the edge of Lake Chillisquaque in July 2010. B. Satellite image of Lake Chillisquaque and the neighboring Pennsylvania Power and Light Corporation.

MATERIALS AND METHODS

Collection

In order to study species diversity and draw conclusions about relationships within these ecosystems, samples were collected from Lake Chillisquaque at various periods throughout the year: September 2009, May 2010, and July 2010. Water samples were collected from one sampling location at the center of Lake Chillisquaque and were obtained from two depths, surface level and 15 feet, using a Van Dorn Sampler. Following collection, samples were kept on ice prior to filtration. At each depth, light intensity was measured using a LI-COR LI-1400 spectrophotometer with underwater sensor in order to determine the quantity of light. Wavelength of light was measured using a Stellar Net EPP 2000 Black Comet CXR model spectroradiometer with a UV-VIS range of 280-900 nm. Measurements demonstrated a degree of light penetration sufficient for survival of cyanobacteria at both depths. In addition, a comparison of light intensity between the two depths showed significant differences in the ambient light quantity as well as differences in the available wavelengths of light between the surface and the depth.

DNA Isolation

Water samples were filtered using 0.22 μm water filters. Samples collected from similar depths and locations were combined and bacterial DNA was extracted using the MoBIO RapidWater DNA Isolation Kit. Two separate primer sets were used in order to PCR amplify the 16S rRNA gene sequences of extracted DNA: CYA106F and CYA781R, a cyanobacterial-specific primer set commonly used when determining phylogenetic relationships among cyanobacterial species, and FD1 and RD1, a more general bacterial primer set (Nübel et al. 1997;

Haverkamp et al. 2009; Weisburg et al. 1990). Amplifying the 16S rRNA regions using these primer sets required PCR conditions of RCAC with denaturation steps 1 and 2 at 94°C for two minutes and one minute, respectively, the annealing step at 55°C for 45 seconds, and the extension step at 72°C for two minutes. RCAC repeats this sequence 25 times and then holds the reaction at 4°C .

Following PCR amplification, samples were cloned and ligated into plasmid DNA of *E. coli* using the TOPO TA cloning kit for sequencing. *E. coli* samples were then incubated at 37°C overnight on LB plates containing kanamycin, creating DNA libraries of bacterial plasmids isolated from Lake Chillisquaque. These plasmids were then isolated and screened using the Zyppy Plasmid Miniprep Kit. Isolated plasmid DNA was then run through gel electrophoresis to verify the presence of plasmid DNA and diluted with 50 μl ddH_20. Samples were sent for Sanger sequencing analysis at the Penn State University Nucleic Acid Facility.

Bioinformatic Analysis

Using the Sequencher program, DNA sequences from the isolated plasmids could be examined. Sequences were aligned at 97% identity, contiged, and assumed to be DNA sequence from the same species, called operational taxonomic units (OTUs). These DNA contigs were then entered into the BLAST program and information on species name, phylogenetic information, accession number, max score, query coverages, and max identity were collected. In order to draw conclusions about the composition of organisms identified, OTUs were combined at the genus level. However, when taking an in-depth look at the OTUs that comprised each genus, OTUs with max identity percentages of equal to or greater than 97% to an identified species were considered to be a strain of the species identified. These were labeled with the

species name indicated by the BLAST database. OTUs with max identity percentages between 97% and 95% to an identified species were considered to be of the same genus of the species identified. These were labeled as the genus name indicated on the BLAST database followed by "sp" to indicate that the genus, but not the species could be specified. OTUs with max identity percentages below 95% to an identified species were considered to be of the same genus as the species identified. These were labeled as "unknown" followed by the family name indicated on the BLAST database.

In order to determine if identified OTUs contained phycoerythrin and/or could chromatically adapt, OTUs were compared at the genus level to genera known to have phycoerythrin-containing and/or CCA-capable species within their genera according to the Pasteur Culture Collection of Cyanobacteria. Those OTUs, and more broadly the genera containing those OTUs, were considered to have phycoerythrin and/or undergo CCA if another known species within their genera could be described by these characteristics.

RESULTS

Light Attenuation

Measurements of the light intensity and available wavelengths of light demonstrated a noticeable difference in the available light between the surface and 15-foot depth in Lake Chillisquaque. Light intensity was approximately 1200 μmol photons/m^2s at the surface and 100 μmol photons/m^2s at the 15-foot depth for all samples. The available wavelengths of light also decreased with increased depth. At the surface, wavelengths of light between 300-900 nm were recorded, with all wavelengths of light within the visible spectrum equally represented. At depth, only wavelengths of light between 450-700 nm were evident, with wavelengths of light around 550 (~ green light) most abundant (Figure 5).

Diversity Estimates

For each depth, 120 sequences were analyzed. Using the CYA primers, the first 40 sequences that were isolated and analyzed showed the greatest proportion of OTUs that had previously not been identified in our samples. The second cohort of 40 samples showed fewer isolates that were previously unidentified, and the third cohort, ranging from isolates numbered 81-120, also included few previously unidentified OTUs. For example, September 2009 depth samples decreased from 7 to 4 to 3 newly identified OTUs with each cohort, May 2010 surface samples decreased from 10 to 1 to 0 new OTUs, and July 2010 surface samples decreased from 10 to 2 to 3 previously unidentified OTUs (Table 1). A gradual tapering off of the proportion of new species identified within each cohort of sequences analyzed indicates that we are approaching sufficient data collection to accurately estimate the amount of diversity in each sample.

Community Composition Across Sample Dates For Surface Samples with CYA Primers

Across September, May, and July, *Oscillatoria*, a filamentous cyanobacteria, was isolated at all three time points from the surface samples using CYA primers. In September, the genera *Oscillatoria* occupied 75.82% of the community, and consisted of only one OTU, which is most closely related to *Oscillatoria limnetica* according to BLAST analysis. Dominance over species composition by *Oscillatoria* on the surface decreased into the summer with 37.62% composition in May and 26.60% composition in July (Figure 6). Notably however, in these samples the percentage of community composition identified as *Oscillatoria* consisted of more than just the OTU identified as *Oscillatoria limnetica*; in both May and July samples a second OTU within the genus *Oscillatoria* was also identified. In May an OTU identified as *Unknown Oscillatoria* was also found, but in a small percentage (0.99%) compared to the OTU identified as *Oscillatoria limnetica* (36.63%). In July, the OTU most closely related to *Oscillatoria sp PCC8927,* which was not observed in other samples, was observed in large percentage in the July sample (16.51%) while the OTU most closely related to *Oscillatoria limnetica* was only responsible for 10.09% of the species composition in July.

The genus *Anabaena*, a filamentous cyanobacteria, was also isolated in all surface samples with increasing percentage from September to July (Figure 6). However, none of the OTUs isolated within the genera *Anabaena* were consistent across sampling times.

Several genera were noted in two of the three samples. Species of *Cylindrospermopsis* and *Dolichospermum*, members of the phylum Cyanobacteria, as well as *Xylophilus*, a proteobacteria, all appear in small proportions in May samples, and slightly larger proportions in July samples; they are not present in the September samples (Figure 6).

A variety of cyanobacterial genera were seen at one sample date, but were not identified in other sample dates throughout the season, for example, *Calothrix* (in September), *Woronichinia* (in May), and *Synechococcus* (in July), among others. Despite this, September and May samples have similar numbers of identified OTUs – 8 and 9 identified, respectively – while the July sample has quite higher OTU diversity at 15 identified isolates (Figure 6). However, some of these OTUs belong to the same genus; diversity increased from September to July with 5 genera present in September, 8 observed in May, and 14 identified in July (Figure 6).

Community Composition Across Sample Dates For Depth Samples with CYA Primers

Comparing the genera identified in depth samples across September, May, and July, several species were consistent throughout. Of the non-cyanobacterial species isolated, *Xylophilus*, a proteobacteria, was present in all samples. It shows increasing dominance over species composition in July samples, accounting for slightly over 83.80% of species identified, compared to the September sample in which it described only 12.70% of the species composition (Figure 7). *Dolichospermum*, a cyanobacteria, was also present in all samples; within this genus the OTU identified as most closely related to *Dolichospermum planctonicum* was observed in highest proportion in the May samples (37.27%), with smaller impact on species composition in September and July at 1.74% and 1.00%, respectively (Figure 7).

Other cyanobacterial genera were isolated at only two of the three time points. *Oscillatoria*, and more specifically the OTU most closely described as *Oscillatoria limnetica*, for example, was found to encompass a large portion of the September samples (55.93%), disappear in the May samples, and return in small proportion in the July samples (4.80%) (Figure 7). *Anabeana* follows a similar pattern, though in much smaller proportions (Figure 7).

Synechococcus, a unicellular cyanobacterial genus, also had an irregular presence in these samples. The OTU most closely related to *Unknown Synechococcus* accounted for 18.18% of the species composition in the May sample, yet was undetected in any other samples. While a second OTU indentified as *Synechococcus sp. PS723* was present in the July sample only, it accounted for a very small portion of the overall species composition (1.00%) (Figure 7).

Although the genera present in each sample changed dramatically between sampling points, the number of OTUs identified – the diversity of species – remained relatively consistent throughout time points, with September having only a slightly higher species diversity than the other two samples; 12 species were identified in September, 7 species were identified in May, and 8 species were identified in July (Figure 7). Some of the OTUs identified, however, belong to the same genus, although genera composition over time followed a similar pattern: 11 genera in September, 7 in May, and 7 in July (Figure 7).

Community Composition between Surface and Depth Samples with CYA Primers

In the September samples, *Oscillatoria* was the only genus that appears in both the surface and the depth samples. In both samples this genera occupied a large proportion of the community composition; 75.82% of the surface sample was comprised of *Oscillatoria*, and was made up of only on OTU, specifically identified as *Oscillatoria limnetica*, whereas 55.93% of the depth sample is comprised of this same OTU. *Anabaena* was also present in both samples, but in much smaller percentages: 3.30% at the surface and 0.85% at depth (Figure 8).

In the May samples, the only cyanobacterial genus with consistent presence between the surface and depth is *Dolichospermum*, though it occupied a larger proportion of the species composition at depth (37.27%) than at the surface (0.99%); in both samples, this genus included

only one OTU, identified as *Dolichospermum planctonicum* (Figure 9). For the non-cyanobacterial genera, *Xylophilus,* a similar relationship was observed. *Navicula*, a eukaryotic genera, was also present in both samples, though it had the opposite surface/depth relationship; it was relatively more abundant at the surface (40.59%) than at depth (20.91%) (Figure 9).

In the July samples, *Xyophilus* had a similar relationship to that of the May samples; it occupied 83.80% of the community at depth and only 6.42% of the community at the surface (Figure 10). Interestingly, *Dolichospermum*, also identified as the OTU most closely related to *Dolichospermum planctonicum*, which in the May samples had larger species composition at depth compared to the surface, occupied a larger proportion of the community at the surface (6.42%) than at depth (1.00%) in the July samples. This relationship with greater community composition at the surface compared to the depth was also true for two other cyanobacterial genera, *Synechococcus* and *Oscillatoria* (Figure 10).

Community Composition with FDRD Primers

Because FDRD primers can identify a broadened spectrum of bacterial diversity, FDRD primer data was analyzed at the phylum level, although isolates identified as belonging to the phylum cyanobacteria were further analyzed to the genus level of taxonomy. Consistently across all data sets for the FDRD primers cyanobacterial species occupied less of the community in surface samples as compared to depth samples (Figures 11-16). For example, in September, at the surface cyanobacterial genera accounted for 49.60% of the community while at depth cyanobacterial genera encompassed 71.29% of the phyla identified (Figures 11 and 12). Also in September, cyanobacterial species occupied the greatest percentage of the community compared to all other sampling time points. The proportion of the community comprised of cyanobacterial

species decreased in May and July, with the smallest portion of the community occupation by cyanobacteria in July (Figures 11-16). For example, at the surface in September, as previously noted, the cyanobacterial species composition was 49.60%, while in May this dropped to 15.21% and in July it remained low at 10.56% (Figures 11,13, and 15).

Comparison of CYA and FDRD Primer Data

Several inconsistencies were apparent between the data from the CYA and FDRD primer sets. In the September depth samples, for example, the FDRD primers indicated the presence of *Raphidiopsis*, *Leptolyngbya*, and *Woronichinia*, all cyanobacterial species, which were not identified in the same sample using CYA primers. In fact, the only genus of cyanobacteria that both primers consistently isolated was *Anabaena* (Figures 8 and 12). In the September surface samples, both primers were in agreement on the presence of *Anabaena*, *Raphidiopsis*, and *Oscillatoria*; however, *Woronichinia*, *Microcystis* and *Leptolyngbya* were isolated using the FDRD primers, but not the CYA primers (Figures 8 and 13). Among May depth samples, *Dolichospermum* was the only consistent cyanobacterial species while *Prochlorales*, *Anabaena*, *Nostoc*, and *Woronichinia* were not found in CYA primer data, but were isolated in the FDRD data (Figures 9 and 14). Interestingly, across all isolates identified with the CYA primers, *Prochlorales*, was never identified (Figures 6 and 7). Furthermore, *Woronichinia*, *Microcystis*, and *Anabaena* were found in surface samples from May according to the data from both primer sets, but the presence of *Calothrix* was inconsistent with CYA data (Figures 9 and 13).

In July samples, a similar, but slightly different, pattern was observed. Depth samples consistently picked up *Oscillatoria*. However, according to the FDRD data, *Oscillatoria* appeared to be the only cyanobacterial species present; the CYA data, on the other hand,

indicated much more cyanobacterial diversity not supported by the FDRD data (Figures 10 and 16).

Finally, the July surface data was the only set of CYA and FDRD data that appeared to demonstrate consistency between the two primer sets. *Anabaena*, *Dolichospermum*, and *Cylindrospermopsis* were consistently found in both FDRD and CYA primer data. However, CYA data also identified additional cyanobacterial genera, for example, *Oscillatoria*, which was not isolated in the FDRD primer sets. Furthermore, the proportion of species diversity among the two primer sets was incongruent. In the July surface data from the FDRD primers, both *Dolichospermum* and *Anabaena* comprised a significant proportion of the cyanobacterial community. However, in looking at the CYA data, *Anabaena* represented a much smaller proportion of the cyanobacterial community than *Synechococcus*, another species undetected in the FDRD data (Figures 10 and 15).

Prevalence of phycoerythrin-containing and CCA-capable Species

Of the species of cyanobacteria isolated, three classes of cyanobacteria were represented: Chroococcales, Nostocales, and Oscillatoriales (Table 2). Each of these classes have species that are reported to contain phycoerythrin and/or undergo the process of complementary chromatic adaptation (CCA). The genus *Synechococcus*, of the class Chroococcales, *Nostoc*, *Tolypothrix*, and *Calothrix*, of the class Nostocales, and *Leptolyngbya*, *Microcoleus*, and *Oscillatoria*, of the class Oscillatoriales all have strains that are known to contain phycoerythrin and/or undergo CCA (Pasteur Culture Collection of Cyanobacteria). Given this information, we can suggest the possibility of species isolated for our environmental samples as containing phycoerythrin and being capable of undergoing CCA. Without culturing samples and completing further analysis

we cannot be positive that the sequences we have isolated are those of phycoerythrin-containing or CCA-capable species; however, linking our isolates with genera of cyanobacteria that do have these characteristics allows us to draw conclusions about the possibility of these characteristics being present in our isolates and sets up a framework for future, more in-depth study of these isolated sequences. Thus, we can begin to draw conclusions about the possible striation of phycoerythrin-containing and CCA-capable species within the water column of Lake Chillisquaque.

Among the September samples, detected species showed no significant difference in abundance of possible phycoerythrin-containing or CCA-capable species between the surface and the depth samples using CYA primers. Interestingly, however, the FDRD primers demonstrated no known phycoerythrin-containing or CCA-capable genera in the depth samples and only a small percentage of these genera in the surface samples. For example, *Leptolyngbya*, *Oscillatoria*, and *Microcystis* were all isolated in the surface samples, but not in the depth samples (Figure 8).

May samples that were isolated using the CYA primers did show some differences in the known phycoerythrin-containing or CCA-capable species. A slightly higher abundance of phycoerythrin-containing or CCA-capable genera were isolated in the surface sample than the depth sample. From the surface sample, *Oscillatoria* (17.62%) could contain strains that contain phycoerythrin and undergo CCA; from the depth sample, *Tolypothrix* (0.91%), *Microcoleus* (0.91%), *Synechococcus* (18.18%) could contain strains that contain phycoerythrin and undergo CCA (Figure 9). Samples isolated with the FDRD primer sets mirror these data (Figures 13 and 14).

Finally, July samples isolated using the CYA primers showed an even higher possible abundance of phycoerythrin-containing or CCA-capable genera in the surface sample compared to the depth sample. *Oscillatoria* (26.60%) and *Synechococcus* (26.61%) were found in the surface sample, while much smaller abundances of *Oscillatoria* (7.70%) and *Synechococcus* (1.00%) were isolated in the depth samples (Figure10). However, the FDRD primers outlined an opposite pattern of phycoerythrin-containing or CCA-capable species. *Oscillatoria* maintained the highest proportion of the cyanobacterial community in depth samples, while *Leptolyngbya* in the surface samples only appeared to comprise a small portion of the species composition (Figures 15 and 16).

Cultures of Species Isolated from Lake Chillisquaque

As part of an independent project, several of our samples from Lake Chillisquaque have been cultured in lab and examined under the microscope. Interestingly, from the July 2010 surface samples, we have been able to isolate and identify species belonging to the genus, *Oscillatoria* (Figure 17). However, we have isolated two different strains of *Oscillatoria*, one with a green-pigmented phenotype and a second with a purple-pigmented phenotype (Figure 17). Further analysis to determine the species identity of these isolates is necessary; however, it is clear that at least two different strains of *Oscillatoria* are present at the surface of Lake Chillisquaque in July.

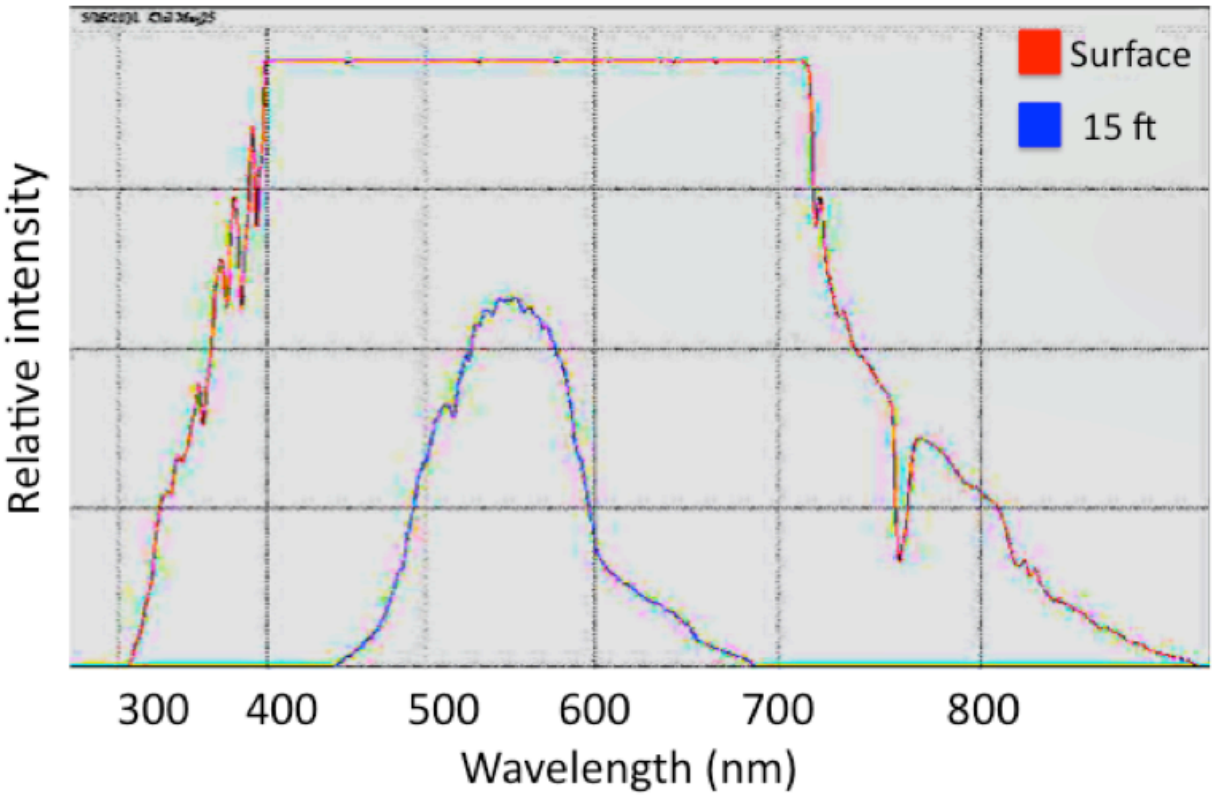

Figure 5. Light intensity and attenuation in Lake Chillisquaque. Spectrometer measurements from May 2010 indicate a narrowing of the visible light spectrum at depth.

Table 1. Number of previously unidentified OTUs isolated in three sequencing cohorts.
The number of isolates that had not been previously identified in our data using the CYA primers decreased with increased sampling and isolation.

sample	# previously unidentified OTUs newly identified		
	1-40 sequences	41-80 sequences	81-120 sequences
July '10 Depth	5	1	2
July '10 Surface	10	2	3
May '10 Depth	5	2	1
May '10 Surface	8	1	0
Sept '09 Depth	7	4	3
Sept '09 Surface	6	1	1

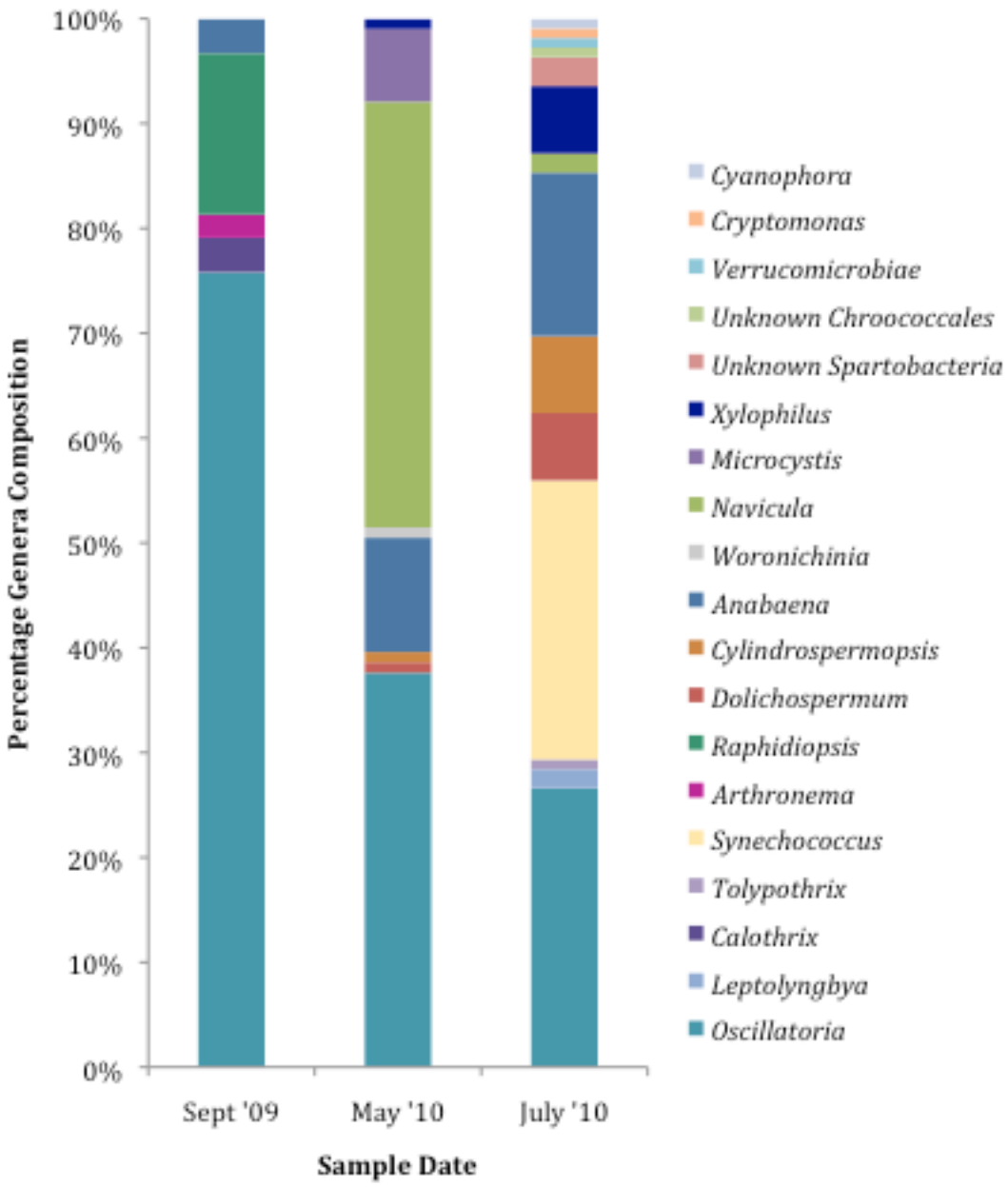

Figure 6. Community composition of surface samples over time using CYA primers. Genera diversity at the surface of Lake Chillisquaque changed throughout the year.

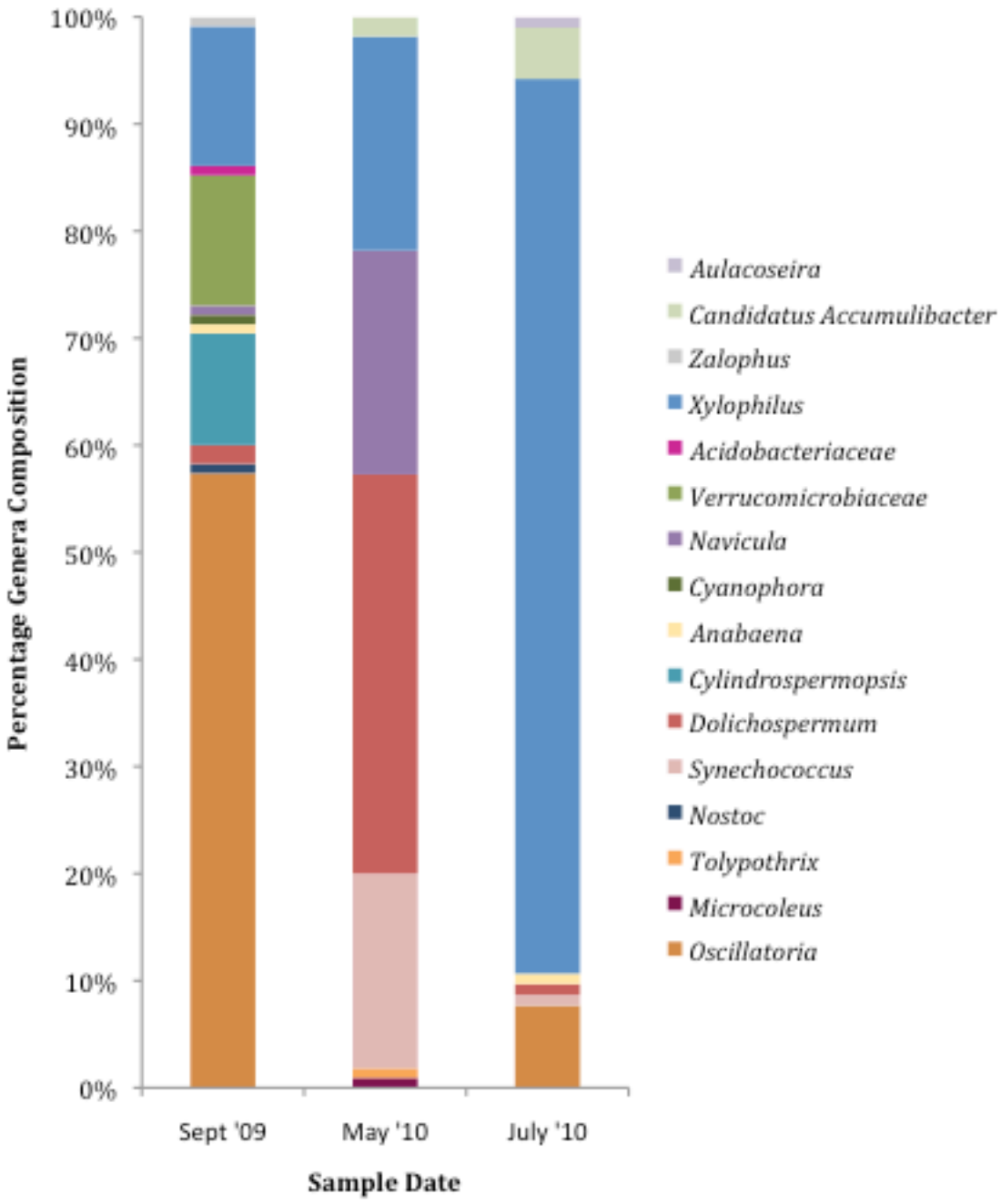

Figure 7. Community composition of depth samples over time using CYA primers. Genera diversity at depth in Lake Chillisquaque changed throughout the year.

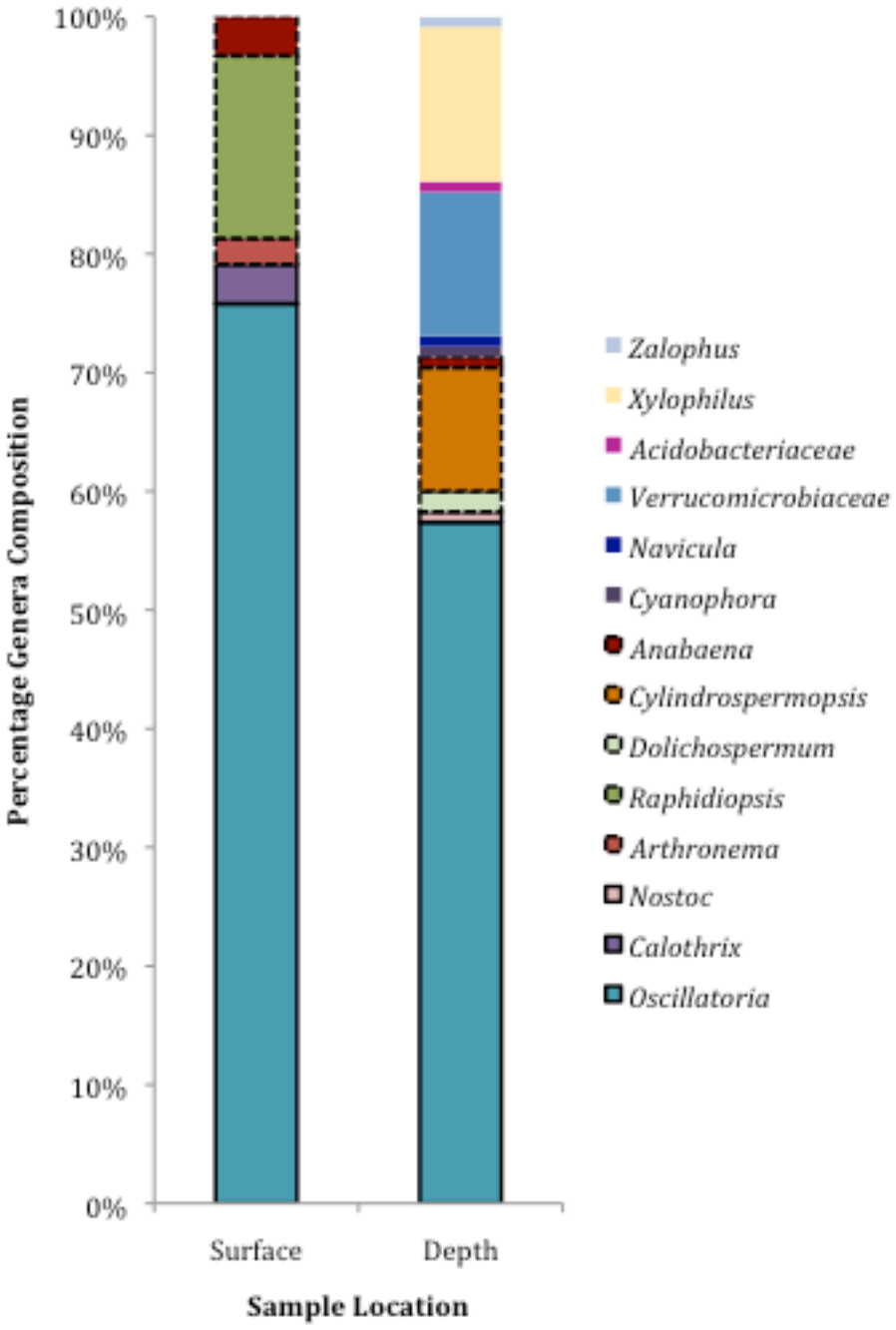

Figure 8. Community composition in September 2009 using CYA primers. The solid border indicates cyanobacterial genera that can contain PE or undergo CCA, the hatched border indicated cyanobacterial genera that are unknown to contain PE or undergo CCA, and no border indicates non-cyanobacterial genera. Genera composition changed between surface and depth samples in September.

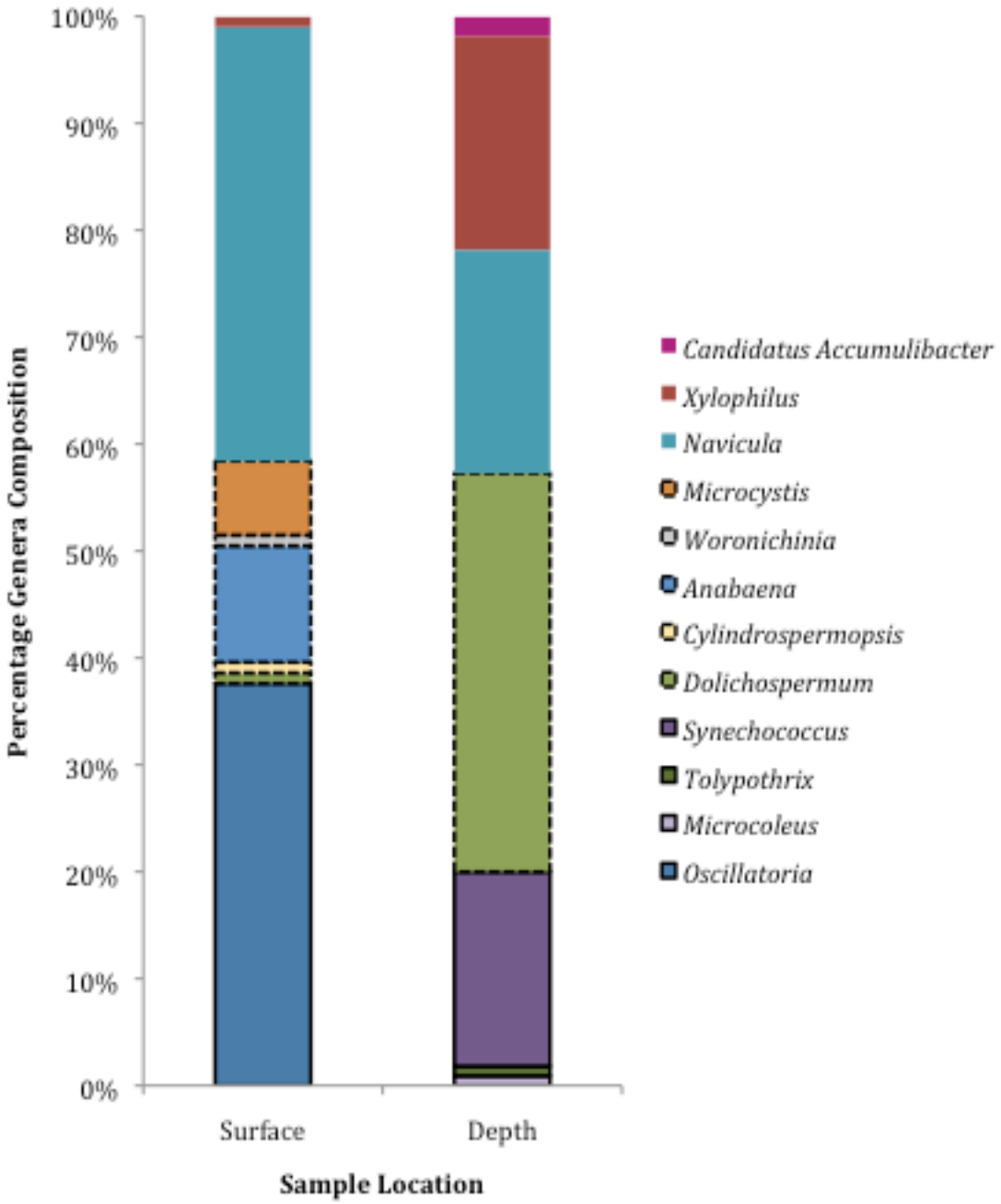

Figure 9. Community composition in May 2010 using CYA primers. The solid border indicates cyanobacterial genera that can contain PE or undergo CCA, the hatched border indicated cyanobacterial genera that are unknown to contain PE or undergo CCA, and no border indicates non-cyanobacterial genera. Genera composition changed between surface and depth samples in May.

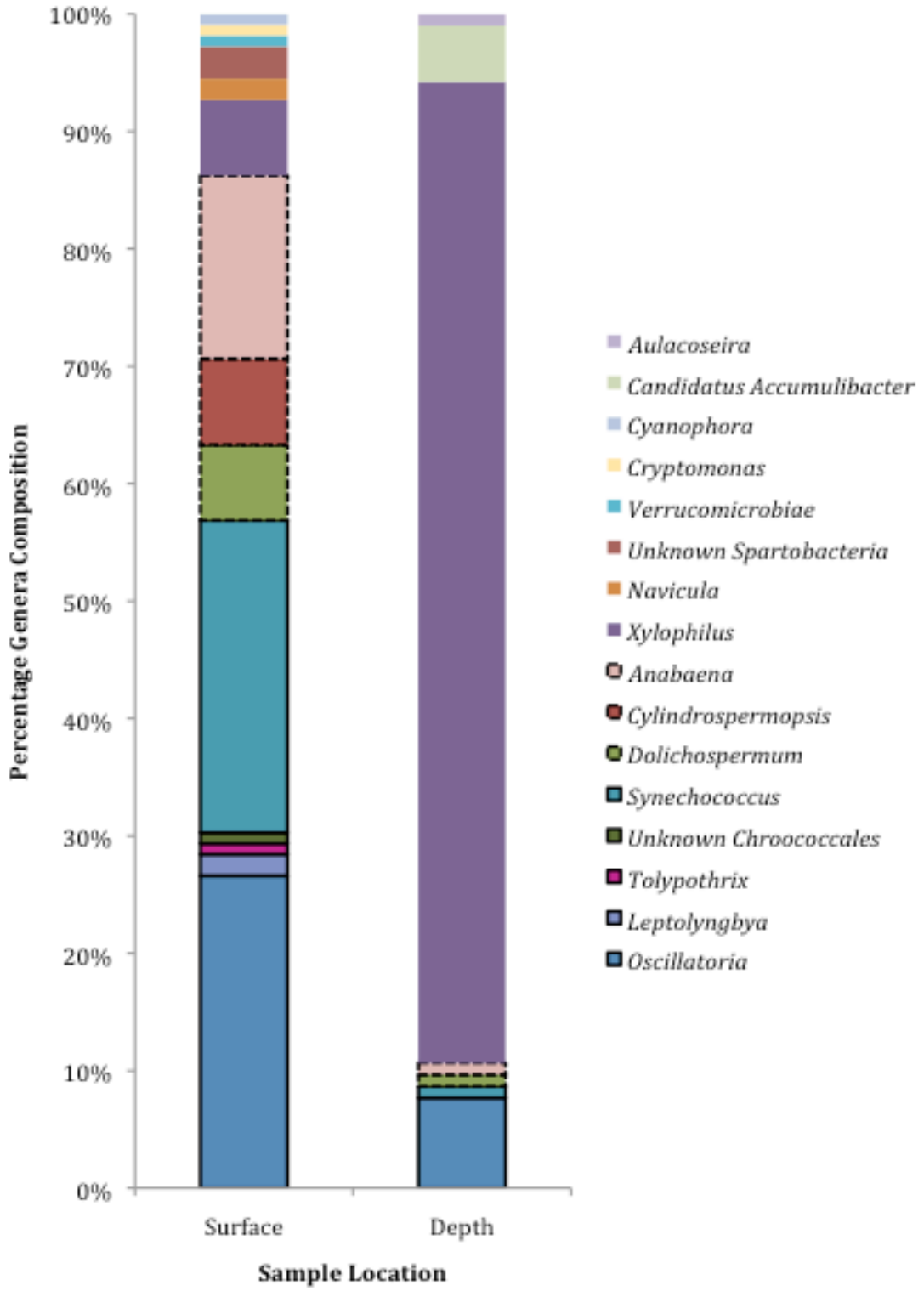

Figure 10. Community composition in July 2010 using CYA primers. The solid border indicates cyanobacterial genera that can contain PE or undergo CCA, the hatched border indicated cyanobacterial genera that are unknown to contain PE or undergo CCA, and no border indicates non-cyanobacterial genera. Genera composition changed between surface and depth samples in July.

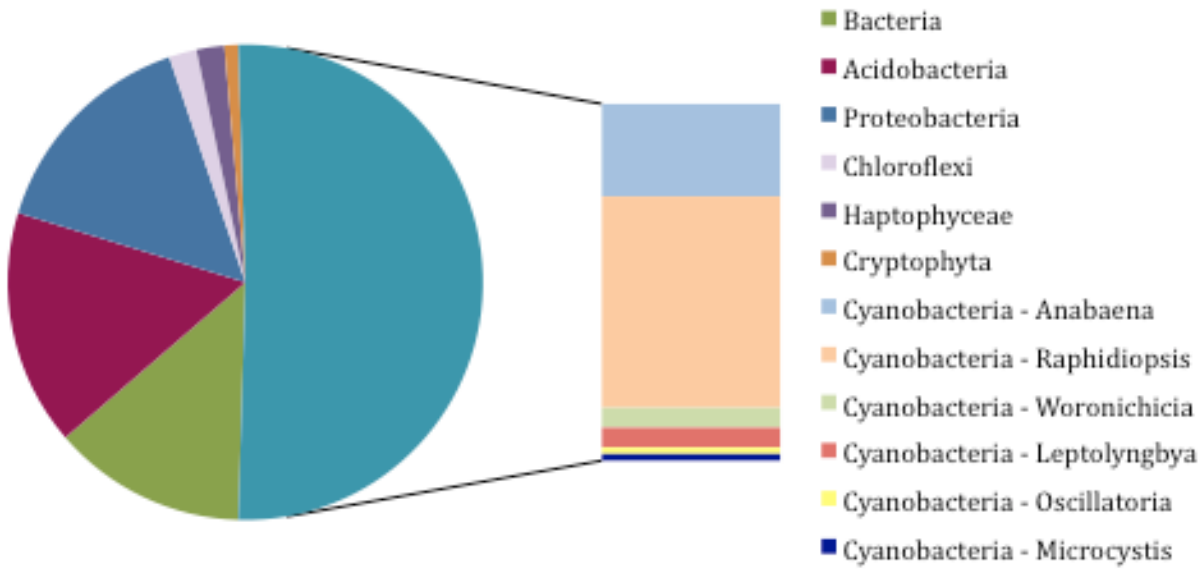

Figure 11. Community composition at surface in September 2009 using FDRD primers. Roughly half of the community consists of genera of cyanobacteria.

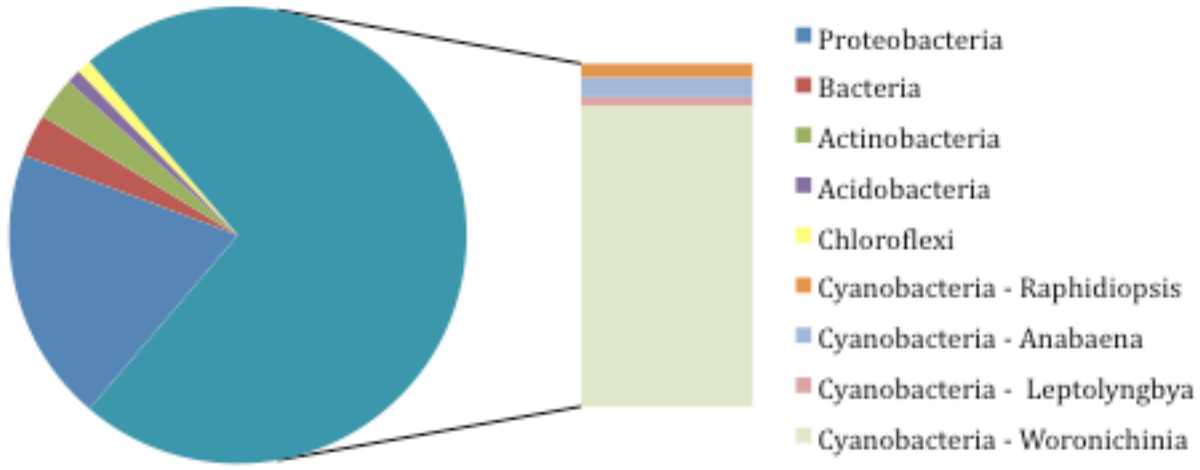

Figure 12. Community composition at depth in September 2009 using FDRD primers. A majority of the community consists of genera of cyanobacteria.

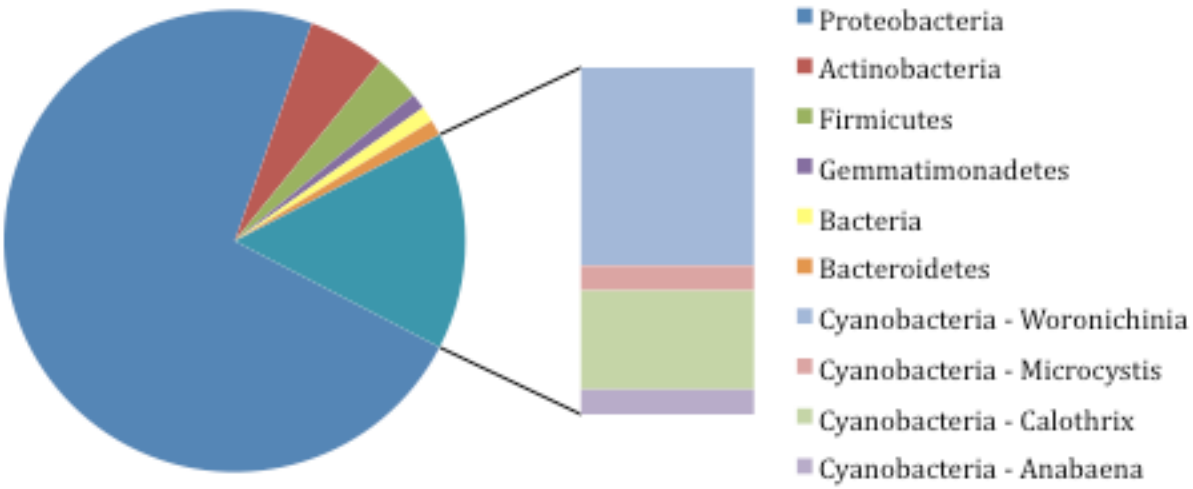

Figure 13. Community composition at surface in May 2010 using FDRD primers. A small portion of the community consists of genera of cyanobacteria.

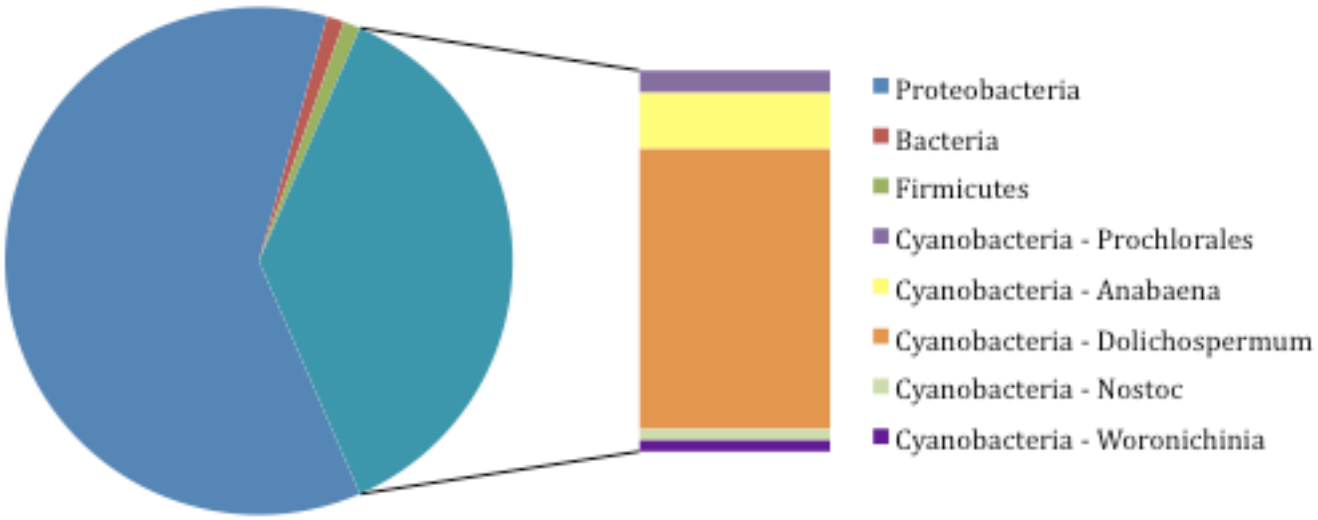

Figure 14. Community composition at depth in May 2010 using FDRD primers. Less than half of the community consists of genera of cyanobacteria.

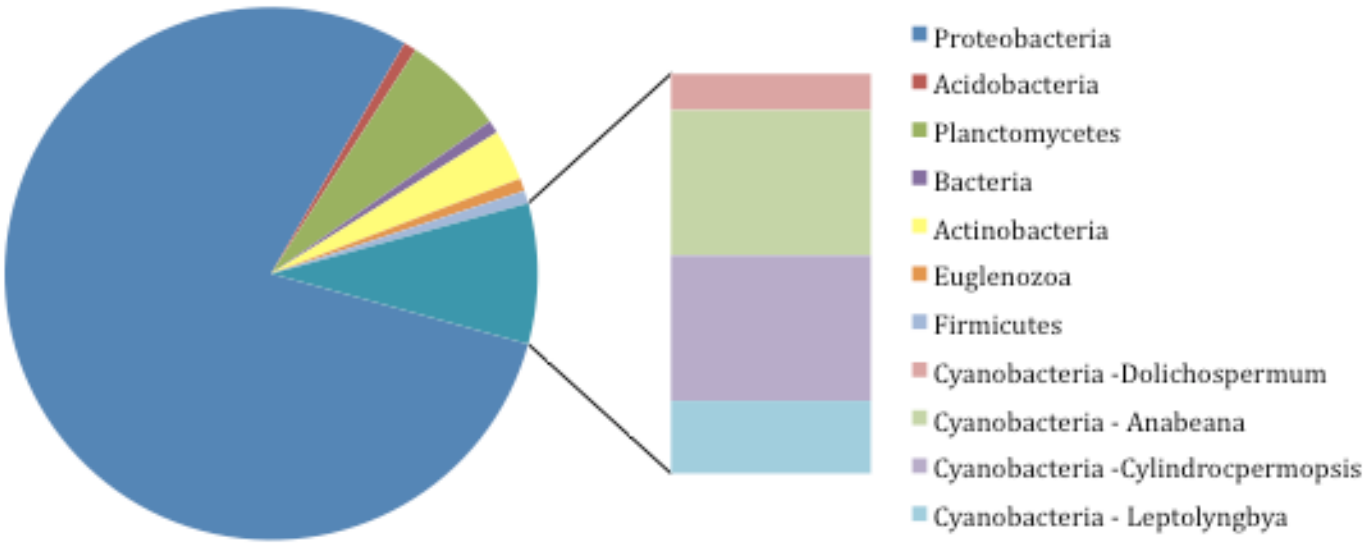

Figure 15. Community composition at surface in July 2010 using FDRD primers. A small portion of the community consists of genera of cyanobacteria.

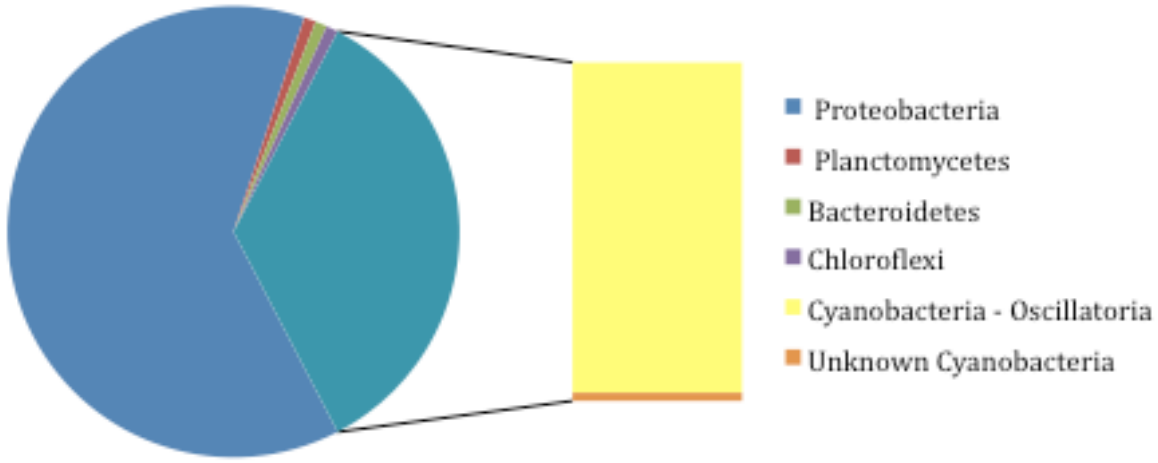

Figure 16. Community composition at depth in July 2010 using FDRD primers. Almost one third of the community consists of genera of cyanobacteria.

Figure 17. *Oscillatoria* isolated from the surface Lake Chillisquaque in July 2010. A. Both green-pigmented and purple-pigmented *Oscillatoria* are identified using microscopy. B. Magnified image of green-pigmented and purple-pigmented *Oscillatoria*.

Table 2. Phylogenetic information for isolated cyanobacteria. Fourteen genera of cyanobacteria have been isolated; these genera belong to three different classes of cyanobacteria.

Domain	Phylum	Class	Genus
Bacteria	Cyanobacteria	Chroococcales	Microcystis
			Synechococcus
			Woronichinia
		Nostocales	Anabaena
			Cylindrospermopsis
			Dolichospermum
			Nostoc
			Raphidiopsis
			Tolypothrix
			Calothrix
		Oscillatoriales	Arthronema
			Leptolyngbya
			Microcoleus
			Oscillatoria

DISCUSSION

Cyanobacteria are photosynthetic organisms containing a variety of light-harvesting proteins that allow for the absorption of various wavelengths of light available within an ecosystem (Wood, 1985). For most species of cyanobacteria, the phycobilisome profile is fixed. However, some species possess the ability to alter phycobilisome composition in order to maximize the uptake of light from the environment; this process is known as CCA (Grossman et al. 2001). In an aquatic ecosystem, available wavelengths of light change with depth in the water column. At the surface, all wavelengths of light within the visible light spectrum (between 380-750 nm) are present; however, with increasing depth, available light narrows to the shorter wavelengths, excluding wavelengths of light that are yellow and red in nature (Stomp et al. 2007). Lake Chillisquaque showed similar patterns of light attenuation with recorded wavelengths of light from 300-900 nm measured at the surface, but only wavelengths of light between 450-700 nm detected at the 15-foot depth (Figure 5). Moreover, while the highest intensity of light at the surface is between 400-700 nm – the wavelengths of light for the entire spectrum of visible light – at depth high intensity light is concentrated around 550 nm, or that of green light (Figure 5). Thus, the available light wavelengths change with depth in the water column of Lake Chillisquaque, and narrow to green light wavelengths at depth.

Given these patterns of light attenuation, we expected to find stratification of cyanobacterial species between surface and depth samples. Species that are phycocyanin-rich, and thus reliant on red light in order to undergo photosynthesis, should be abundant in surface samples where red light is plentiful; they should be rare in depth samples where red light is scarce. Species that are phycoerythrin-rich could be found in both surface and depth samples;

however, we expect them to occupy a higher percentage of the species composition at depth, where other cyanobacterial species are unable to flourish. Finally, we expected to find a higher number of chromatically-adapting species at the depth than at the surface. Because all wavelengths of light in the visible spectrum are available at the surface, it is less likely that a species will need to alter phycobilisome composition, deeming CCA less necessary for surface-dwelling species. However, with increased depth, and, thus, greater light attenuation, the ability to change phycobilisome composition in order to harvest whatever wavelength of light is most available is necessary. Therefore, we predicted that CCA species are likely to have increasing abundance with increasing depth in the water column.

Comparing surface and depth samples from Lake Chillisquaque in September, May, and July we found several variations in the community structure, both between location in the water column and time of sampling. In looking at genus composition across time, we found a cycling trend among genera. Several genera occupied a large percentage of the community during a specific sample date, but had much smaller presence at the other two sampling time points. In the surface samples, for example, *Oscillatoria* was prominent in the September community analysis, but showed decreasing presence throughout the summer months (Figure 6). Other genera were recorded at only two sample points, and still others could only be detected at one sample point throughout the year. This is consistent with theories of ecosystem cycling that describe periods of decreased growth of bacterial and cyanobacterial species, most often during the cool, winter months (November – March) followed by bursts in bacterial and cyanobacterial species abundance during warm, summer months (April – October) (Porter et al. 1996). Each species of cyanobacteria has optimal nutrient and temperature requirements, thus it is reasonable to assume that each species will have a period throughout each year when it is most abundant, followed by

a gradual tapering off of species abundance as the environment moves away from these optimal conditions as the year progresses (Callieri et al. 2007).

Data from the phyla composition of Lake Chillisquaque using the FDRD primers also shows a similar cycling pattern with cyanobacteria encompassing the greatest percentage of the community in September while tapering off in community composition throughout the summer months (Figure 11-16). Again, this is consistent with theories on ecosystem cycling; many organisms have characteristic periods of the year that are more favorable for their growth, while other periods of the year lead to near dormancy of the population (Porter et al. 1996).

Little genera consistency was also noted between surface and depth samples taken at the same time point. Many genera with presence in surface samples were unrecorded in depth samples. Of those genera that were found at both locations in the water column, often they occupied a much greater percentage of community at either the surface or at the depth. For example, in the May samples, *Dolichospermum*, is the only cyanobacterial genera recorded at both locations in the water column, yet it has a much greater presence at depth than at the surface (Figure 9).

Given the change in genera observed throughout the water column, some environmental factor must be playing a role in the stratification of species in Lake Chillisquaque. Because light attenuation also changes throughout the water column, and because cyanobacteria rely on light for energy, it is reasonable to assume that this could be the factor that is altering the cyanobacterial community structure between surface and depth samples. In fact, research has shown that light attenuation is responsible for more than half of the picocyanobacterial diversity differences observed throughout the water column in freshwater lakes of varying sizes and

locations (Pick 1991). In order to connect changes in the composition of genera throughout the water column in Lake Chillisquaque with light attenuation, however, we must analyze the phycobilisome composition of the identified species at surface and at depth to determine if they contain phycoerythrin and can undergo CCA. Given our current data, we cannot identify the specific phycobilisome composition with the species identified; instead, we can only classify isolated OTUs as belonging to genera with known strains of cyanobacteria that have been identified to contain phycoerythrin and/or undergo CCA.

Given this data, we were unable to prove that a higher proportion of CCA-capable species exists at depth. Instead, we found a greater number of OTUs that belong to genera with known complementary chromatic adaptors in the surface samples. However, without further analysis of each OTU, the conclusions drawn from these data are not sufficient to support or refute our original hypothesis on CCA-capable species diversity. We cannot be certain, for example, that the strains we have observed contain phycoerythrin or are capable of undergoing CCA; we can only know that they are closely related to known strains that are phycoerythrin-containing and CCA-capable. It could be possible, in fact, that though we identified several cyanobacterial strains from the surface samples that are related to phycoerythrin-containing and CCA-capable strains, that very few of our surface OTUs actually possess these functional characteristics. The opposite could be true of our depth OTUs – though fewer isolated sequences were related to species that are known to contain phycoerythrin or undergo CCA, it is possible that some of the specific strains we identified might be phycoerythrin-containing and CCA-capable. Further analysis, either by culturing each OTU and testing for phycoerythrin and CCA or by using primers that are specific to genes that encode phycoerythrin proteins, is necessary in order to draw appropriate conclusions.

We were, however, able to show that genera of cyanobacteria likely to undergo CCA or contain phycoerythrin coexist with species that cannot undergo CCA and contain phycocyanin only. For example, in the surface samples from September 2009, all identified OTUs were of the phyla cyanobacteria. Almost 80% of the genera of cyanobacteria identified were recognized as being closely related to strains of cyanobacteria that can undergo CCA and contain phycoerythrin, thus they were assumed to have to contain phycoerythrin and undergo CCA. Yet, 20% of the genera identified in this sample did not fit these classifications, thus, they were assumed to contain phycocyanin only (Figure 8). Accordingly, the coexistence of phycocyanin and phycoerythrin-containing species is evident. This is consistent with models of cyanobacterial species diversity that predict that, due to the formation of environmental niches around light wavelength availability, cyanobacterial species with variations in phycobiliprotein composition can coexist in order to maximize the use of all available light (Stomp et al. 2004). These models are supported by research on Baltic Sea cyanobacterial diversity that concluded that strains of *Synechococcus* contain a variety of pigmentation phenotypes in order to maximize their intake of environmental light and, thus, create a variety of environmental niches (Haverkamp et al. 2009).

Other projects in our lab have also illustrated this coexistence of cyanobacteria with differing pigmentation phenotypes. From the July 2010 surface samples obtained from Lake Chillisquaque, lab cultures revealed the coexistence of both green-pigmented, and thus phycocyanin-containing, and purple-pigmented, and thus phycoerythrin-containing, cyanobacterial strains assumed to be of the genera *Oscillatoria* (Figure17). Thus, coexistence of cyanobacteria with differing phycobilisome composition does occur at the surface of Lake Chillisquaque. To date, we have been unable to identify these specific strains of *Oscillatoria*; however, some of the data we have acquired using the CYA primers indicates that one of these

isolates could be the OTU we have identified as closely related to the species *Oscillatoria limnetica*.

Beyond these conclusions, however, one flaw in our current data is the inconsistency in composition of genera of cyanobacteria derived from our two sets of primers. While some genera were detected using both sets of primers, others were isolated using only one of the two primer sets. This phenomenon is known as primer bias, and it is possible that due to primer bias one (or both) of our primer sets is less likely to anneal to the 16S rRNA regions of some classes of cyanobacteria, thus overemphasizing the presence of some genera of cyanobacteria, deemphasizing the presence of other genera of cyanobacteria, and ignoring the presence of still other genera of cyanobacteria. In order to accurately draw conclusions about the species composition at surface and at depth in Lake Chillisquaque, is it necessary that we use primers that are impartial to all genera of cyanobacteria.

Given the current inconsistency between the FDRD and CYA primer sets, we cannot be positive that we are accurately drawing conclusions about species composition in Lake Chillisquaque. The next step in our research, therefore, is to generate new primers that do not selectively measure species diversity. Our current data will guide us in this attempt as we have shown the types of genera of cyanobacteria that can inhabit Lake Chillisquaque at different time points throughout the year. Knowing that these species exist in Lake Chillisquaque provides a starting point in the production of new primers for further analysis. Furthermore, in the future we hope to use primers that can isolate sequences encoding phycoerythrin and phycocyanin proteins. This will allow for us to draw more precise conclusions about the light-harvesting characteristics of isolated species and it will aid our understanding of species composition as a result of light attenuation throughout the water column. Finally, we also hope to use metagenomics analysis

and high-throughput sequencing techniques, which will eliminate the need for cloning as well as the likelihood for primer bias.

Despite these future directions, our data still allow us to draw some significant conclusions about the community structure of Lake Chillisquaque. We have found that species composition changes markedly throughout the year as well as throughout the water column, and we have also shown that these changes can be correlated with light attenuation throughout the water column. Finally, we have also demonstrated the coexistence of cyanobacterial species with variations in pigmentation phenotype and phycobilisome composition in Lake Chillisquaque at the surface and at depth in September 2009, May 2010, and July 2010.

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